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We have isolated a monoclonal antibody binding to oestradiol with high affinity (3.7 nM), and exhibiting a better than 1000-fold selectivity in binding to other steroids. A high affinity antibody with good specificity is essential for the accurate determination of circulating oestradiol levels. To date, conventional hybridoma technology has not yielded a reagent of sufficiently high affinity and specificity for this ligand. The aim of this study was to investigate whether such a reagent was accessible through the engineering of antibodies on the surface of filamentous phage. Antibodies were isolated from a large repertoire of single chain Fv fragments (scFv) derived from non-immunised human donors, with selection and screening procedures biased to favour those binding to free oestradiol. This resulted in an antibody with nanomolar affinity for oestradiol, while affinities for related steroids are in the micromolar range. This antibody appears to be unique among anti-steroid monoclonal antibodies in lacking a 'blind-spot'. Our results demonstrate that phage display can provide solutions to problems that have so far proved intractable using conventional hybridoma technology.

Anti EGP-2 phage antibodies as a tool for identifying novel colon tumor antigens. [Abst. 223] Rob C. Roovers[a], Adriaan de Bruine[a], Bart-Jan Kroesen[b], Wijnand Helfrich[b], Loe de Leij[b], Jan-Willem Arends[a], Hennie R. Hoogenboom[a], *[a]CESAME at the Department of Pathology, University Hospital Maastricht, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands, [b]Department of Clinical Immunology, University Hospital Groningen, Oostersingel 59, 9713 EZ Groningen, The Netherlands.*

Antibody phage technology provides a new tool for the identification of novel cancer antigens. We aim to identify novel tumor antigens on colorectal cancers by selecting naïve and patient-based phage antibody repertoires on colon tumor cells. With fine-tuned substraction and depletion selection regimens, we expect to be able to find phage antibodies that can identify completely novel tumor antigens. To assess the crucial conditions for selection, we first developed a model system based on a well-characterized tumor associated antigen, the epithelial glycoprotein-2 (EGP-2).

We cloned two anti EGP-2 antibodies (MOC-31 and MOC-161) as single-chain variable fragments (scFv's) displayed on phage by amplification of the V-genes using RT-PCR and assembly in scFv format. Screening for binding clones was performed by means of an ELISA using recombinant baculovirus-expressed EGP-2. For the MOC-161 antibody, 5% of the recombinant antibodies bound antigen; for the MOC-31, it was necessary to perform two rounds of selection of a mini-library to identify binding scFv's. The specificity of both scFv antibodies was confirmed by immunohistochemistry on a panel of EGP-2-positive and -negative cell lines.

Secondly, we show that phages displaying the MOC-31 scFv antibody can indeed be selected from a large excess of non-relevant phages by panning on colon CaCo2 cells. A comparison of cell panning versus MACS and FACS substraction selections will be presented. These model cell selections will enable us to establish the conditions needed for selection of novel tumour antigens in colon cancer, using large naïve phage antibody repertoires, or repertoires derived from the tumour-draining lymph nodes of patients with colon cancer.

Novel immunogen structures and immunization strategies induce antibodies to conserved epitopes of cannabinoids. Steven M. Rosen, Raymond Hui, Linda Vitone, Sheria Yeh, Cassandra Cottell, Ellen Bender, Salvatore J. Salamone, *Roche Diagnostic Systems, Inc., Somerville, N.J. 08876, USA.*

Immunogens that are capable of inducing monoclonal antibodies to conserved epitopes on structurally-related small molecules are important for generating immunodiagnostic tests for drugs. For example, anti-cannabinoid antibodies that have broad specificity for tetrahydrocannabinol (THC) metabolites are highly desirable for use in assays for this abused drug. Recognition of common core structure(s) between THC and its major metabolites is most likely a pre-requisite for the specificity of such antibodies. We have used a sequential immunization protocol that we call Multiple Immunogen Medicated Epitope Selection or *'Mimes'* wherein we use three cannabinoid immunogens. One of these immunogens is a novel benzpyran molecule that mimics a cannabinoid core structure and selectively induces antibodies that possess high crossreactivity to six major cannabinoid metabolites. The antibodies derived by the combined use of these immunogens and this immunization protocol possess significantly higher cross-reactivities to cannabinoid metabolites than those obtained by conventional immunization strategies that rely on one or two immunogens. Furthermore, these antibodies are able to detect the presence of cannabinoid metabolites in clinical samples at a higher rate than other antibodies derived from the use of conventional immunization procedures.

Engineering antibodies for tumor targeting using phage display. Robert Schier[a], G. Adams[b], K. Marshall[a], A. McCall[b], L. Weiner[b], M. Bookman[b], James D. Marks[a], *[a]Departments of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA, [b]Fox Chase Cancer Center, Philadelphia, PA, USA.*

A human scFv which binds to the tumor antigen c-erbB-2 was isolated from a non-immune phage antibody library by panning on a c-erbB-2 ECD (extracellular domain) coated polystyrene tubes. We determined binding kinetics and tumor targeting properties from the primary isolate, C6.5, and compared these results to those obtained using murine hybridoma derived 741F8 scFv'. Mutant C6.5 phage antibody libraries were created by chain shuffling and randomizing light chain CDR3 (VLCDR3). Affinity driven selections against c-erbB-2 ECD delivered scFv with improved K_d and changes in k_{on} and k_{off} compared to C6.5. With one exception, a slower k_{off} is responsible for the improved affinity in all the mutants. C6L1 (a light chain shuffled clone) and C6H2 (a heavy chain shuffled clone) have a 2–3-fold slower k_{off} and a slightly faster k_{on} compared to C6.5. We isolated mutant scFv from the VL-CDR3 randomized library which were present with a low frequency in the library and an even lower k_{off} than the chain

shuffled mutants. After optimizing the elution condition for the phase selection, scFv mutants were obtained with a 16-fold improved K_d compared to C6.5. Sequencing of these mutant scFv revealed conserved amino acids within the VLCDR3 as well as dominant amino acid (AA) changes. Furthermore, an alanine scan of the heavy chain CDR3 was performed to identify AA involved in binding of C-erbB-2. Based on the alanine scan data, we created four new phage display libraries by randomizing four amino acids at a time of the heavy chain CDR3 (VHCDR3). We isolated a monomeric scFv fragment from one of these libraries with a 100-fold improved affinity compared to C6.5 Amino acid positions with high mutation frequency were identified. Combining the results based on sequence, structure and kinetics of mutant scFv, we constructed three new VHCDR3 libraries. These libraries differ in length of VHCDR3, AA position randomized and frequency with which certain AA occur. We are in the process of making combinations of several different mutant scFv in a monovalent and bivalent form. The broad range of these mutant scFv will be utilized to examine the effect of affinity, binding kinetics and valency on the tumor targeting properties of antibody constructs.

Protein engineering: redesigning of the antibody combining site.
X. Sidiropoulou, A.R. Rees, *School of Biology and Biochemistry, University of Bath, UK.*

In the *de novo* design of antibody specificity, the aim is to define a set of CDR residues which is able to bind a predefined antigen. A method by which such a sequence set can be derived using a simple Monte Carlo docking procedure and parsimonious use of residue types will be described.

The starting point was a well-characterized monoclonal antibody (Gloop2) whose x-ray structure had been determined in this laboratory and whose specificity for residues 57–84 (the loop peptide) of hen-egg lysozyme (HEL) was well characterised. The target specificity was enkephalin/morphine, whose X-ray and NMR structures were also known. Using the theoretical design procedure, a docked complex between an all-alanine version of the Gloop 2 combining site and enkephalin was constructed and after reconstruction of side chains, thirteen contact residues (eight in the heavy and five in the light chain) were identified as candidate positions for mutations. Each position was mutated to one of six residue types, selected on the basis of both chemical redundancy and allowing for the fact that certain residues occur with high frequency in antibody combining sites. The resulting library was computationally screened using a simple force field and ten low energy complexes were selected. The ten candidate antibodies were expressed as ScFv fragments on the surface of filamentous phage and the mini-library screened for binding to biotinylated enkephalin. After three rounds of selection binding was observed and, when the DNA from the eluted binding phage was sequenced, it was found to match one of the predicted mutation set. The ScFv DNA of this selected design (GlaMor) was subcloned as a cassette from the phage to an expression vector, expressed as soluble protein in the periplasm of bacteria and purified using metal chelate affinity chromatography.

The binding and specificity of GlaMor will be described. Further mutations to improve the specificity and affinity of this new design will also be presented.

Rational mutation of a catalytic antibody.
Lee C. Smith, Anthony R. Rees, *School of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK.*

Antibody 20G9 which catalyses the hydrolysis of phenyl acetate to acetic acid and phenol was raised against phenyl phosphonate, an analog of the presumed transition state of the reaction. The catalytic formation of phenol is measured spectrophotometrically at 270 mm and gives a rate acceleration over background of 10^4. [1]. Kinetic studies have implicated the involvement of tyrosine(s) in the mechanism, suggesting an acyl-tyrosyl intermediate [2]. A model of 20G9 scFv was generated using AbM (Oxford Molecular Ltd) and was used as a basis on which to select residues likely to be involved in the catalytic mechanism. Twelve residues: Y (x7), R (x3), D and H were selected based upon the previous kinetic evidence, their proximity to the combining site groove and their relative reactivity. The effect of mutagenesis of these residues on the catalytic parameters of the antibody will be discussed. In parallel, expression and purification of high levels of 20G9 scFv will allow crystallisation of the antibody complexed to the transition state analog. Mutations based upon the antibody model and crystal structure should provide insight into the catalytic mechanism and may lead to improved kinetic performance.

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Generation of an estradiol specific anti-body by phage display.
Rodger G. Smith, Ralph Abraham, John Link, Colleen Venti, Michael Darsley, *IGEN, Inc., 16020 Industrial Drive, Gaithersburg, MD 20877, USA.*

Current diagnostic immunoassays for steroid hormones such as estradiol and estriol utilize polyclonal rabbit serum. From a production and quality control standpoint, monospecific non-serum derived reagents would be beneficial. Due to the high degree of structural relatedness between steroid hormones generating highly specific, high affinity monoclonal antibodies has been difficult. We utilized a phage display system in an attempt to isolate high affinity antibodies against the steroid hormone estradiol. Using PCR, the VH and VK antibody repertoires were amplified from cDNA prepared from spleens of mice hyper immunized with estradiol-BSA. The VH and VK fragments were linked together with a 6-bp spacer (2 amino acids) and cloned into a modified version of the phagemid vector pCANTAB5. Modifications included the addition of an octapeptide (FLAG) and HIS_6 for immunological detection and IMAC purification, respectively, of expressed antibody. The shortened linker between VH and VK allows expression of the antibody repertoire on the phage surface as a bivalent single-chain Fv or diabody. Phage were generated from a library comprising 1×10^7 clones and panned twice on a solid phase coated with estradiol-BSA. Clones were screened for production of estradiol specific diabody using an electro-